

Purification of Porcine Enterokinase by Affinity Chromatography

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A method is described for the purification of porcine enterokinase by affinity chromatography with *p*-aminobenzamidine as the ligand. Purification was completed by immunoadsorption with antisera raised to components binding non-biologically to the gel. The final enterokinase preparation was 2.3 times more active than the most active preparation previously described.

A method has been described for the purification of porcine enterokinase (enteropeptidase, EC 3.4.21.9) in which the principal fractionating step was gel filtration on a bed (200 cm × 6 cm diam.) of Sephadex G-100 and a bed (600 cm × 4 cm diam.) of Sephadex G-200 (Maroux *et al.*, 1971; Baratti *et al.*, 1973a). These authors did not state whether their preparation was free of other enzymic activities, particularly the intestinal disaccharidases. The present paper describes an improved preparative procedure for enterokinase by the use of affinity chromatography. The ligand selected was *p*-aminobenzamidine, which has a K_i for enterokinase of 2.0×10^{-5} M (Geratz, 1969).

Materials and methods

A commercially available extract of enterokinase, prepared by the method of Kunitz (1939) from hog duodenal content, was used as the starting material (Miles Laboratories Ltd., Stoke Poges, Slough, Bucks. SL2 4LY, U.K.). *p*-Aminobenzamidine was kindly supplied by May and Baker Ltd., Dagenham, Essex RM10 7XS, U.K. Pig kidney aminopeptidase-M was obtained from Rohm G.m.b.H., Darmstadt, Germany, and crystallized trypsinogen from Boehringer Corp. (London), London W5 2TZ, U.K.

Preparation of substituted Sepharoses for affinity chromatography. The coupling procedure was similar to that described by Cuatrecasas (1970). Portions of washed Sepharose 4B were activated with 150–200 mg of CNBr per g of packed gel. The Sepharose was coupled with the appropriate 'spacer arm', either γ -amino-*n*-butyric acid or glycylglycine at a concentration of 2 mmol/ml of gel. The substituted gel was washed in sequence with cold distilled water and three alternate 300 ml portions of 0.05 M-sodium phosphate buffer, pH 7.6, and 0.05 M-sodium citrate buffer, pH 3.6, each containing 1 M-NaCl. Then 3 mmol of *p*-aminobenzamidine was coupled to 50 g batches of the substituted gel with 6 mmol of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide. The

amount of ligand covalently bound to the gel was quantified by the method of Failla & Santi (1973).

Preparation of antisera for immunoadsorption.

(i) Rabbit anti-(porcine kidney aminopeptidase-M). Five New Zealand white rabbits were each inoculated subcutaneously with 1.5 mg of the commercial enzyme preparation distributed over four sites. An intravenous boost of about 300 μ g was given at 6 weeks, and serum was obtained 2 weeks later. (ii) Rabbit anti-(porcine maltase/glucoamylase). Material isolated during the preparative procedure which contained no demonstrable enterokinase activity, but potent maltase and glucoamylase activity, was used as the antigen. Intravenous boosting was performed at 3 and 6 weeks, and 10 days before each subsequent bleed. Substitution of Sepharose 4B with the antisera was carried out as described by Gospodarowicz (1972). Immunoglobulin was precipitated with 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ and resolubilized in 0.9% NaCl and dialysed against 0.1 M-NaHCO₃ containing 0.5 M-NaCl. The dialysed material was added to an equal volume of packed gel previously activated with CNBr (150 mg/g of Sepharose). The pH was adjusted to 8 and the slurry stirred overnight at 4°C. The gel was washed with cold distilled water and the hyperosmotic buffers as described above.

Enzyme assays. The enterokinase assay was based on the improved method of Baratti *et al.* (1973b). First, 0.1 ml of enterokinase solution and 0.2 ml of 70 mM-sodium succinate buffer, pH 5.6, were pre-incubated at 30°C. To this was added 0.25 ml of a trypsinogen solution (1 mg/ml in 1 mM-HCl made 2.2 mM with CaCl₂). The final calcium concentration was 1 mM. The activation was stopped after 30 min by the addition of 20 μ l of 2.5 M-HCl. Then 0.2 ml of this mixture was assayed for trypsin at 30°C with *N*- α -benzoylarginine ethyl ester as substrate (Schwert & Takenaka, 1955). The commercial trypsin standard (Miles Laboratories) was 50% active by weight assessed by the method of Hixson & Nishikawa (1973). This was taken into account when calculating the specific activity of enterokinase, which was expressed as nmol of trypsin produced/min per mg of

enterokinase protein. Protein was determined by the method of Lowry *et al.* (1951). Crystalline bovine serum albumin (Hopkin and Williams, Chadwell Heath, Essex, U.K.) was used as the standard.

Aminopeptidase activity was determined with L-leucine *p*-nitroanilide as substrate; 0.1 ml of test solution was added to 3 ml of 0.05 M-sodium phosphate buffer, pH 7.2, in a cuvette maintained at 30°C. Then 0.1 ml of substrate (0.025 M) was added and the change in E_{405} was recorded. The specific activity was expressed as nmol of nitroaniline produced/min per mg of protein. Disaccharidase and glucoamylase activities were measured by the two-stage method of Dahlqvist (1968); a unit of disaccharidase activity is that hydrolysing 1 μ mol of disaccharide/min. The activity was expressed in units/ml.

Preparative procedure. Batches (20 g) of crude enterokinase were solubilized in 1 litre of ice-cold 10 mM-Sorensen's phosphate buffer, pH 7.6. Insoluble material was removed by centrifugation at 15000g for 2 h at 4°C. The supernatant was dialysed against 10 litres of the same buffer at 4°C, with three changes, for 48 h. The non-diffusible material was cleared by mixing with a little Celite filter aid (BDH, Poole, Dorset, U.K.) and re-centrifuging for 15 min. All chromatographic procedures were carried out in a cold-room at 4°C. The clear brown supernatant was loaded on to a DEAE-cellulose ion-exchange column pre-equilibrated with the buffer. Elution of enterokinase was achieved by a linear 0–200 mM-NaCl gradient from 2 \times 200 ml reservoirs of buffer, with the peak activity emerging at 70 mM-NaCl. Fractions containing enterokinase were pooled and dialysed against 50 mM-Tris-HCl buffer, pH 8.5.

The initial substituted gel used for the affinity chromatography of enterokinase incorporated γ -amino-*n*-butyric acid as the spacer arm. The ligand concentration was estimated to be 2.2 μ mol/ml of gel. A column of this gel of bed dimensions 8.5 cm \times 1.5 cm diam. was washed extensively with 50 mM-Tris-HCl, pH 8.5. The solution containing enterokinase was pumped through the bed and the eluate collected in 7.5 ml fractions. After loading, the column was washed for 16 h with the buffer. The enzyme peak was eluted with 15 ml of 50% (v/v) ethylene glycol in 0.5 M-Tris-acetic acid buffer, pH 5.6, containing 1 M-NaCl. Selected fractions were assayed for enterokinase and aminopeptidase activities. The four major enterokinase-containing fractions were pooled and dialysed against 50 mM-Tris-HCl, pH 8.5, at 4°C.

A 45 ml bed of rabbit anti-(porcine aminopeptidase-M)-substituted Sepharose 4B was equilibrated with 50 mM-Tris-HCl, pH 8.5, containing 0.2 M-NaCl. The binding capacity of the gel was approx. 0.6 mg of antigen protein per ml of gel. The dialysed material was made 0.2 M with NaCl, pumped through the bed and the eluate retained. The column could be

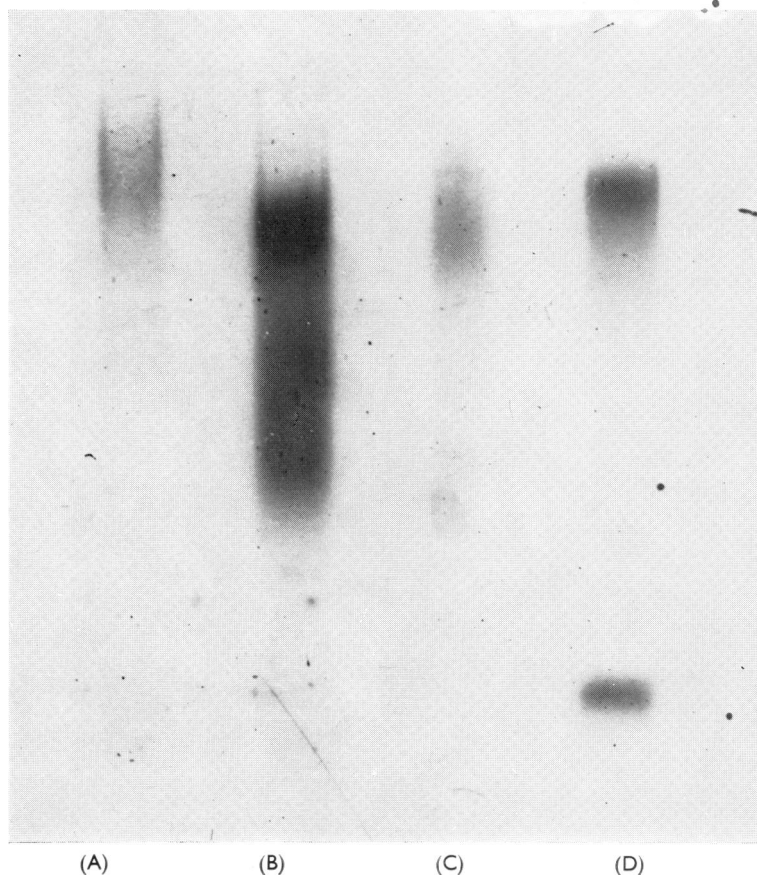
reconstituted and used repeatedly by washing with 6 M-guanidine hydrochloride.

The second substituted gel used for affinity chromatography incorporated glycylglycine as the spacer arm. The ligand concentration was estimated to be 3.6 μ mol/ml of gel. A short column of bed dimensions 7.5 cm \times 1.5 cm diam. was packed and equilibrated with 50 mM-Tris-HCl, pH 8.5. The material containing enterokinase, previously dialysed against the same buffer, was pumped through the column and collected in 9 ml fractions; no enterokinase activity was lost during loading. The column was then washed extensively with the series of buffers indicated in Fig. 1. Enterokinase was eluted as before with 15 ml portions of the ethylene glycol-1 M-NaCl solution, pH 5.6. Enterokinase, maltase and glucoamylase activities of eluted fractions were determined. Disaccharidase activities were measured after dialysis to remove the Tris (Swamnithan & Radhakrishnan, 1970). Fractions 221–224 were pooled for the final stage.

A 200 ml bed of rabbit anti-(porcine maltase/glucoamylase)-substituted Sepharose was equilibrated with 0.2 M-NaCl in 50 mM-Tris-HCl, pH 8.5. The binding capacity of the gel was 0.7 unit of maltase/ml and 0.8 unit of glucoamylase/ml. The pooled enterokinase dialysed against the same buffer was pumped through the immunoabsorbent. The eluate was retained. The enzyme was concentrated on a short DEAE-cellulose column equilibrated with 10 mM-sodium phosphate buffer, pH 6.5. Sharp elution of the enzyme was achieved with 0.175 M-sodium phosphate, pH 6.5. The enzyme was stored in 10 mM-sodium phosphate buffer, pH 7.0, at –20°C.

Results

The specific activity of the crude supernatant was 5.6 units. The γ -amino-*n*-butyrate-*p*-aminobenzamidine-substituted gel removed over 90% of the total aminopeptidase activity; 32% of the total maltase and glucoamylase activities were removed during this stage, together with other disaccharidases and alkaline phosphatases. The remaining aminopeptidase was completely removed by immunoabsorption. The two steps achieved a 22-fold purification compared with the crude supernatant (specific activity 123.5 units). The second affinity chromatography on the glycylglycine-*p*-aminobenzamidine gel removed another 67% of the total maltase and glucoamylase activities and a further fourfold purification resulted. Removal of the residual 1% of the maltase and glucoamylase was completed by immunoabsorption. The yield was 464 μ g (14.3%), the final specific activity 3640 units and the total purification 650-fold. Enterokinase activity was such that 1.0–1.5 ng could be detected by the assay system.



EXPLANATION OF PLATE I

5% Polyacrylamide-gel electrophoresis at pH8.9

Anode was at the bottom. Gel buffer was 0.37M-Tris-HCl, pH8.9, and reservoir buffer was 0.19M-glycine-0.025M-Tris, pH8.4. The gel was run at a constant voltage of 150V for 90min at 4°C. It was stained with 0.4% (w/v) Coomassie Brilliant Blue R in 12.5% (w/v) trichloroacetic acid, aq. 10% (v/v) acetic acid and destained in 10% (w/v) trichloroacetic acid. (A) Porcine enterokinase (25 μ g, specific activity 3640 nmol/min per mg); (B) maltase/glucoamylase antigen (32 μ g); (C) crude supernatant (35 μ g, specific activity 5.6 nmol/min per mg); (D) aminopeptidase-M (35 μ g).

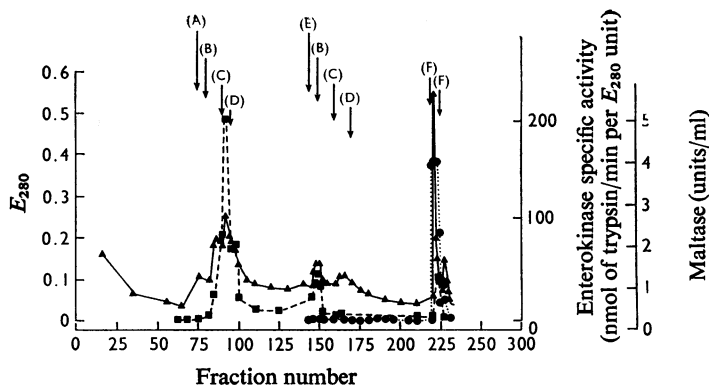


Fig. 1. Elution of porcine enterokinase from glycylglycine-*p*-aminobenzamidine-substituted Sepharose

The specific activity of enterokinase was expressed as nmol of trypsin produced/min per E_{280} unit; 9 ml fractions were collected every 20 min. Enterokinase was eluted by 50% (v/v) ethylene glycol in 0.5 M-Tris-acetate, pH 5.6, containing 1 M-NaCl. Δ , E_{280} ; \bullet , enterokinase specific activity; \blacksquare , maltase activity. The arrows indicate the use of the following eluents: (A) 0.1 M-Tris-HCl, pH 8.4; (B) 0.2 M-Tris-HCl, pH 8.4; (C) 0.25 M-Tris-HCl, pH 8.4; (D) 0.2 M-Tris-HCl, pH 8.4, overnight; (E) 0.3 M-Tris-HCl, pH 8.4; (F) 50% (v/v) ethylene glycol, pH 5.6.

The preparation was free of any other enzyme activity tested, including aminopeptidase, maltase, isomaltase, lactase, sucrase, cellobiase, trehalase, glucoamylase and alkaline phosphatase. The appearance on polyacrylamide-gel electrophoresis is shown in Plate 1.

Discussion

p-Aminobenzamidine has been used as a ligand for the purification of thrombin (Schmer, 1972) and trypsin (Hixson & Nishikawa, 1973) by affinity chromatography. The present study shows that it may also be used for the purification of enterokinase. The resolution of the substituted gel is at least four times better when glycylglycine rather than γ -amino-*n*-butyric acid is used as the spacer arm, presumably owing to a decrease in non-specific hydrophobic binding along the lines suggested by O'Carra (1974). The combination of immunoabsorption and affinity chromatography in a purification procedure is particularly applicable when non-biological binding occurs, since contaminants can be displaced from the gel and used as antigens. Successful adsorption of intestinal aminopeptidase by antisera to kidney aminopeptidase-M indicates that these enzymes are immunologically similar. The γ -amino-*n*-butyrate-linked gel was used during the development of the purification procedure for enterokinase. The superior resolution of the glycylglycine-linked gel may allow this step to be omitted with improvement in yield.

The dispersed appearance of the purified enzyme on gel electrophoresis may be due to microheterogeneity, since enterokinase has been shown to be a

glycoprotein (Yamashina, 1956; Baratti *et al.*, 1973a), and the appearance was similar whether the gel was stained for protein or carbohydrate. Maroux *et al.* (1971) expressed the activity of their enterokinase preparation as nmol of trypsinogen activated/30 min per mg. For simplicity we have defined the activity as nmol of trypsin produced/min per mg. The specific activity of porcine enterokinase purified by affinity chromatography as described here is calculated to be 2.3 times greater than the most active preparation previously reported (Baratti *et al.*, 1973a).

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